



Development of Conventional and Real-Time PCR Assays to Detect *Alternaria burnsii* in Cumin Seed

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Abstract

Alternaria burnsii is the causal agent of cumin blight, a seed-borne disease of economic concern for all cumin growing areas. Current detection and identification methods for the pathogen are based on visual examination of morphological features, which are time-consuming and laborious. The present study describes conventional and real-time PCR assays for rapid and accurate detection of *A. burnsii* in cumin seeds. Based on sequence differences in *Alternaria* allergen a1 (Alt a1) gene, two primer pairs, Ab35/326 and AB177/403, were designed for conventional and real-time PCR assays, respectively. Both primer pairs amplified the expected target PCR fragment from *A. burnsii* genomic DNA. The sensitivity of conventional PCR with primer pairs Ab35/326 was 1 pg of genomic DNA and allowed the detection of pathogen in cumin seeds samples with 0.2% infestation rate. Real-time PCR assay was highly sensitive and allowed the quantification of 0.1 pg pathogen DNA. Also, this assay confirmed the presence of pathogen in cumin seeds up to 0.1% infestation level. The standard curve ($r^2 = 0.99$) showed a good correlation between fungal DNA quantities and Cq values. The specificity of primer pairs was confirmed by the absence of amplified product with DNA of related fungi species and healthy plant tissue. The assays developed in this study provide a rapid and sensitive tool for the detection and quantification of *Alternaria burnsii* in cumin seed.

Keywords Cumin · *Alternaria* blight · Pathogen detection · Conventional PCR · Real-time PCR

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Entwicklung von konventionellen und Real-Time-PCR-Assays zum Nachweis von *Alternaria burnsii* in Kreuzkümmelsamen

Zusammenfassung

Alternaria burnsii ist der Erreger der Kreuzkümmelfäule, einer durch Samen übertragenen Krankheit, die für alle Anbaubereiche von Kreuzkümmel von wirtschaftlicher Bedeutung ist. Die aktuellen Nachweis- und Identifikationsmethoden für den Erreger basieren auf der visuellen Untersuchung morphologischer Merkmale, die zeitaufwändig und mühsam ist. Die vorliegende Studie beschreibt konventionelle und Real-Time-PCR-Assays für den schnellen und genauen Nachweis von *A. burnsii* in Kreuzkümmelsamen. Basierend auf Sequenzunterschieden im Alternaria-Allergen a1 (Alt a1) Gen wurden zwei Primerpaare, Ab35/326 und AB177/403, für konventionelle bzw. Real-Time-PCR-Assays entwickelt. Beide Primerpaare amplifizierten das erwartete Ziel-PCR-Fragment aus der genomischen DNA von *A. burnsii*. Die Sensitivität der konventionellen PCR mit den Primerpaaren Ab35/326 betrug 1 pg genomische DNA und ermöglichte den Nachweis von Krankheitserregern in Kreuzkümmelproben mit einer Befallsrate von 0,2 %. Der Real-Time-PCR-Assay war hochsensitiv und erlaubte die Quantifizierung von 0,1 pg pathogener DNA. Außerdem bestätigte dieser Test das Vorhandensein von Krankheitserregern in Kreuzkümmelsamen bis zu einer Befallsrate von 0,1 %. Die Standardkurve ($r^2=0,99$) zeigte eine gute Korrelation zwischen DNA-Mengen und Cq-Werten. Bei der Verwendung von DNA verwandter Pilzarten und gesundem Pflanzengewebe wurde kein amplifiziertes Produkt gebildet, wodurch die Spezifität der Primerpaare bestätigt werden konnte. Die in dieser Studie entwickelten Assays stellen ein schnelles und sensitives Verfahren für den Nachweis und die Quantifizierung von *Alternaria burnsii* in Kreuzkümmelsamen dar.

Schlüsselwörter Kreuzkümmel · Alternaria-Fäule · Erregernachweis · Konventionelle PCR · Real-Time-PCR

Introduction

Cumin (*Cuminum cyminum* L.) is an economically important spice crop, widely cultivated in the world. Cumin seeds are used in medicine and cosmetic products as well as adding condiments and flavors to food. Turkey is an important producer of cumin, with a contribution of about 6% of the world production (Divakara Sastry and Anandaraj 2013).

Cumin blight caused by the fungus *Alternaria burnsii* is a major biotic constraint affecting cumin production worldwide. The pathogen causes necrotic black-brown lesions on leaves, seeds and stems, and eventually death of the whole plant. The emergence of cumin blight increases in cloudy weather after flowering and causes yield losses up to 70% (Holiday 1980). Similarly, *Alternaria alternata* has been reported to less commonly cause cumin blight (Divakara Sastry and Anandaraj 2013; Özer and Bayraktar 2015). Both pathogens are seed-borne and survive in the diseased plant residues over long periods (Divakara Sastry and Anandaraj 2013).

Breeding of resistance cultivars, seed treatments, and the use of disease-free plant material are proposed as the most effective means to control seed-borne plant pathogens. However, there is currently no accepted effective methods available for controlling cumin blight in Turkey. Cumin cultivars in our country are quite limited and resistant varieties to the disease are unavailable. Also, there are no fungicides registered for use in controlling the disease. Thus, the use

of healthy seed is the best preventative control measures against this pathogen.

Accurate and rapid identification of the pathogens responsible for disease is essential to improve the efficiency of disease control methods. The currently available methods for detection and identification of *Alternaria* spp. are based on visual examination of morphological features developing on agar or moist blotter papers (Simmons 2007). However, conventional methods are time-consuming, labor intensive and require extensive training. Polymerase chain reaction (PCR) has become a powerful tool for the identification and detection of plant pathogenic fungi. Phylogenetically informative genes such as Internal transcribed spacers (ITS), glyceraldehyde-3-phosphate dehydrogenase (gpd), endopolygalacturonase (endoPG) gene, β -tubulin, mitochondrial small-subunit (mt SSU), and *Alternaria* allergen a1 (Alt a1) gene have been widely used to distinguish *Alternaria* spp. and related fungi species (Pryor and Bigelow 2003; Hong et al. 2005; Udayashankar et al. 2012; Lawrence et al. 2013). PCR-based assays with species-specific primers have been developed for detection and identification of some *Alternaria* species (Johnson et al. 2000; Konstantinova et al. 2002; Guillemette et al. 2004; Pavón et al. 2010; Udayashankar et al. 2012; Leiminger et al. 2015). The aim of the present study was to develop conventional and real-time PCR assays for specific and rapid detection of *A. burnsii* in cumin seeds based on *Alternaria* allergen a1 (Alt a1) gene sequences.

Table 1 List of fungal species used in this study

Fungal species	Number of isolate	Host plant
<i>Alternaria burnsii</i>	35	Cumin
<i>Alternaria alternata</i>	4	Cumin
<i>Alternaria infectoria</i>	1	Cumin
<i>Ulacladium</i> sp	1	Cumin
<i>Embellisia</i> sp	2	Cumin
<i>Fusarium oxysporum</i> f. sp. <i>cumini</i>	5	Cumin
<i>Fusarium acuminatum</i>	1	Cumin
<i>Fusarium solani</i>	1	Cumin
<i>Fusarium equiseti</i>	1	Cumin
<i>Macrophomina phaseolina</i>	1	Cumin
<i>Rhizoctonia solani</i>	1	Cumin
<i>Fusarium oxysporum</i> f. sp. <i>ciceris</i>	4	Chickpea
<i>Sclerotinia minor</i>	1	Jerusalem artichoke
<i>Sclerotinia sclerotiorum</i>	1	Jerusalem artichoke
<i>Macrophomina phaseolina</i>	1	Jerusalem artichoke
<i>Verticillium</i> sp	1	Goji berry
<i>Stemphylium vesicarium</i>	1	Asparagus

Materials and Methods

Fungal Cultures

Isolates of *Alternaria burnsii* and other fungi species used in this study are presented in Table 1. Pure single-spore cultures of all isolates were maintained on PDA (Potato Dextrose Agar) at 23 °C with a 12:12-h light-dark photoperiod, and stored at 4 °C. Morphological identification of *Alternaria* spp. from cumin was carried out according to Simmons (2007). The cultures were grown on PDA, PCA or V88 media under alternating temperature and light regimes to induce sporulation and examined at 25× with a stereo microscope. The identifications were also confirmed by sequencing of the ITS and Alt a1 gene regions (Özer and Bayraktar 2015).

Seed Samples

Naturally infected seed samples were collected from seven cumin fields located in Ankara and Konya provinces of Turkey in 2016. The seed samples were sterilized with 1% NaOCl for 1 min, washed in sterile distilled water and dried with paper towels. The seeds from each field were milled

into fine powder using lab mill and stored until used for DNA extraction. The pathogen infection in each seed lot was also confirmed by standard blotter method. To determine the detection limits of PCR assays, the powder of infected seeds picked up under stereomicroscope was pooled with the clean seed powder in increasing proportions to generate eight infestation levels (50%, 30%, 10%, 2%, 1%, 0.4%, 0.2%, 0.1% infestation level) and then used for DNA extraction.

DNA Extraction

Seed and fungal DNA were extracted using GeneJET Plant Genomic DNA extraction kit (Thermo Scientific) following the manufacturers directions. Mycelium or seed samples (approximately 100 mg) was ground in liquid nitrogen using micropestle. The fine powder was used for DNA extraction. The quality of DNA was confirmed by running on 0.8% agarose gel. DNA was quantitated using a NanoDrop-1000 spectrophotometer (Thermo Scientific), and stored for use in future studies. PCR efficiencies were determined by generating standard curves using ten-fold serial dilutions of pathogen DNA ranging from 10 ng to 0.01 pg with plant DNA from healthy cumin seed.

Table 2 Description of primer sets used for PCR assays of *Alternaria burnsii*

Primer	Sequence 5'—3'	PCR product (bp)
Alt for	Alt a1 gen	ATG CAG TTC ACC ACC ATC GC
Alt rev	(Hong et al. 2005)	ACG AGG GTG AYG TAG GCG TC
AB177	Real-time PCR	CCC TCA ACT TCA CCT GCG G
AB403		GGA AAC ACC CTG GCA GAT GT
AB35	Conventional PCR	GAG TCT CGC CAG GAC AAC T
AB326		CGT AGG TCG CAT CGG TGT

reaction containing 20 ng DNA, 0.2 μ M of primer pair, and 5 $\times$ HOT FIREPol Blend Master mix (Solis BioDyne, Estonia). PCR amplifications were carried out in a thermal cycler programmed as follows: 12 min at 94 °C; 40 cycles at 94 °C for 30 s, 60 °C for 30 s, 72 °C for 45 s and 72 °C for 7 min. The PCR products were separated electrophoretically on 2% agarose gels in 1 $\times$ TAE buffer and visualized by staining with ethidium bromide (Sambrook et al. 1989).

Real-time PCR reactions were performed using LightCycler System (Roche Diagnostics Corp., USA). PCR reaction was carried out in 20 μ L reaction mix containing 5 μ L sample DNA (10 ng), 0.25 μ M of primer pair, and 2 $\times$ FastStart Essential DNA Green Master Mix (Roche Diagnostics Corp., USA). The thermal cycling conditions were as follows: 10 min at 95 °C; followed by 35 cycles at 95 °C for 20 s, 65 °C for 20 s, 72 °C for 20 s and data acquisition at 83 °C for 3 s. After the final PCR cycle, a melting curve was constructed by measuring the fluorescence continuously when heating from 65 to 97 °C at the rate of 0.2 °C per s. Control reactions contained water and plant DNA instead of template DNA. The C_q values against the base 10 logarithm of the quantity of each standard were used to determine the amount of sample DNA in each assay well. The obtained data were analyzed by using the LightCycler® 96 software (version 1.1). There were three replicates for each DNA sample, and the experiment was repeated twice. PCR products were also analyzed by electrophoresis on 2% agarose gel in TAE buffer.

Results

Sequence Analysis of Alt a1 Gene

Alt a1 gene was selected as a target for specific primer design. PCR amplification with Alt for/rev primer yielded a single product of approximately 440 bp. Sequence alignments of the pathogen isolates and the reference isolates representing *Alternaria* species-groups described by Lawrence et al. (2013) revealed high level of identity between the isolates of the same species. *Alternaria burnsii* isolates were placed in a unique group different from other *Alternaria* spp. and related fungi species with a bootstrap value of 99% (Fig. 1). Sequences of representative *A. burnsii* isolates were deposited in the GenBank database under accession numbers KJ526180-KJ526181.

Primer Design for Conventional and Real-Time PCR Assays

Two PCR primer pairs were constructed based on the DNA sequences of Alt a 1 gene in order to specifically detect *A. burnsii* from pure culture and infected seeds (Table 2).

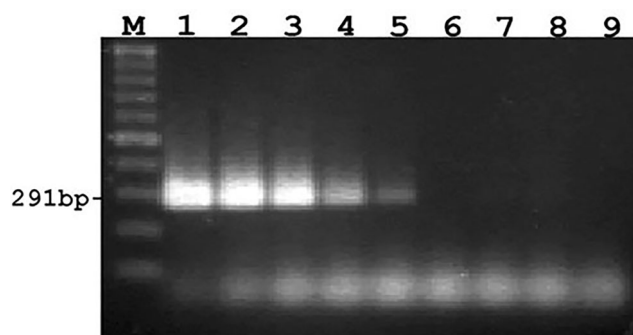


Fig. 2 Sensitivity of conventional PCR assay using Ab35/326 primers and different concentrations of *A. burnsii* genomic DNA as template (lane M: 100-bp DNA ladder; lane 1: 10 ng; lane 2: 1 ng; lane 3: 0.1 ng; lane 4: 0.01 ng; lane 5: 1 pg; lane 6: 0.1 pg; lane 7: 0.01 pg; lane 8: healthy plant DNA; lane 9: water control)

Primer pairs Ab35/326 were designed for conventional PCR, while the other primer pairs AB177/403 were developed for real-time PCR analysis. Primer sequences were searched using BLAST analysis, and determined not to show homology with existing sequence in GenBank. The specificity of PCR primers was assessed on DNA extracted from a collection of pure fungal cultures.

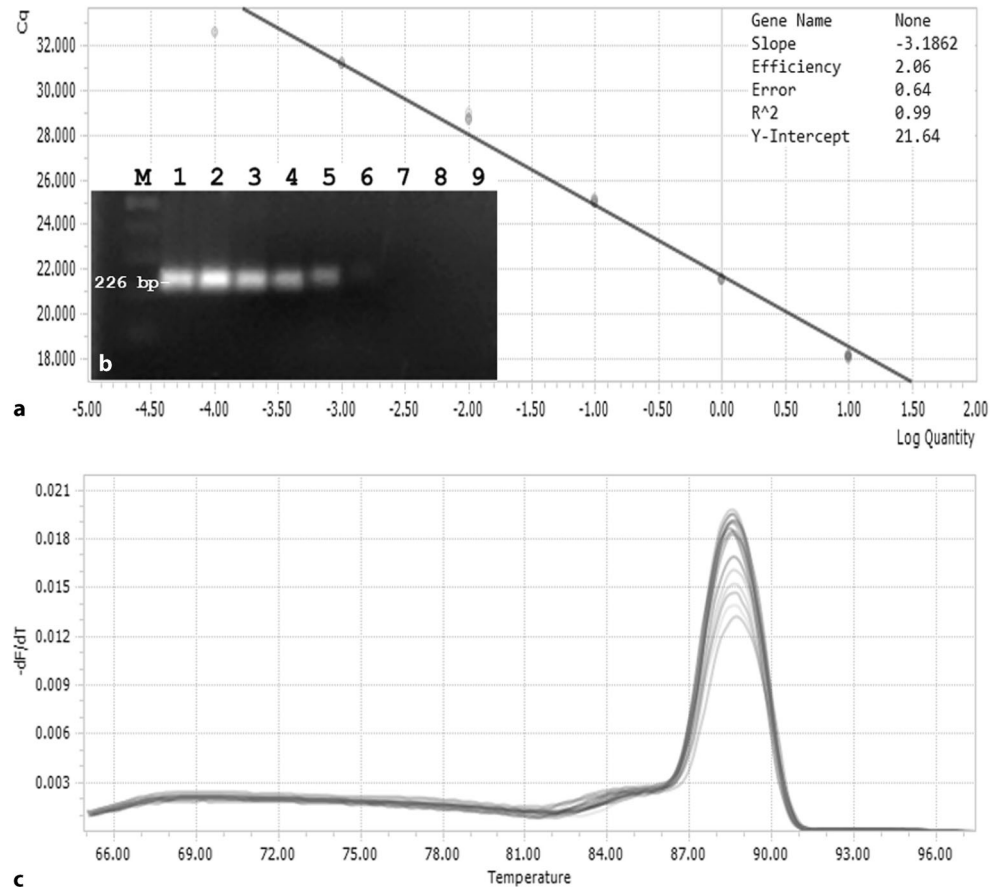
The primer pairs Ab35/326 allowed to selectively amplify a 291-bp product from the DNA extracted from all tested *A. burnsii* isolates. No amplification products were obtained from the other fungal species and cumin plant tissues. To determine the detection limit of conventional PCR assay with Ab35/326 primer pairs, a dilution series of pathogen DNA ranging from 10 ng to 0.01 pg was tested. The detection limit of the primer pairs was detected as 1 pg of genomic DNA (Fig. 2).

The primer pairs AB177/403 designed for real-time PCR assay yielded a single PCR product of 226 bp in size from *A. burnsii* genomic DNA. The results indicated that the detection sensitivity was 0.1 pg of pathogen DNA. The correlation coefficient between the log values of known DNA concentrations in the dilution series and C_q values showed a linear relation ($r^2=0.99$). The slope and intercept values of the standard curve were 3.18 and 21.64, respectively. C_q values of the dilution series ranged from 18.05 to 32.56. Melting curve analysis showed the presence of a single PCR product in reaction with a dissociation temperature of approximately 88.5 °C. The presence of a PCR product of the correct size was also verified by gel electrophoresis. No amplification peak was observed with genomic DNA of the other fungi species and healthy plant samples (Fig. 3).

Determination of *A. burnsii* in Naturally Infected Cumin Seeds

To determine the detection limits of conventional and real-time PCR assays in naturally infected cumin seeds, PCR

Fig. 3 Detection and quantification of *A. burnsii* by real-time PCR analysis using the primer pair Ab177/403. Standard regression lines used for real-time PCR assay to quantify *A. burnsii* genomic DNA (10 ng to 0.01 pg) (a). Agarose gel electrophoresis of qPCR products of 10-fold dilution series of the pathogen DNA starting from 10 ng (lane M: 100-bp DNA ladder; lane 1: 10 ng; lane 2: 1 ng; lane 3: 0.1 ng; lane 4: 0.01 ng; lane 5: 1 pg; lane 6: 0.1 pg; lane 7: 0.01 pg; lane 8: healthy plant DNA; lane 9: water control) (b). Melting curve analysis showing the presence of a single melting peak at the end of the PCR amplification (c)



assays were performed in the sample mixtures prepared by mixing different proportions (50% to 0.1% infestation level) of infested seed and healthy seed powder. Both PCR assays amplified PCR products of the expected size from DNA extracts of seed powder mixtures. Conventional PCR could detect the presence of *A. burnsii* DNA in the seed powder mixture with 0.2% infestation level at least, while the detection limit of real-time PCR was found to be 0.1% infestation level. Cq values of seed powder mixtures with different infection levels ranged from 18.8 to 32.04. Also, the validity of both PCR assays has been tested on seven seed samples obtained from different cumin fields with a range of infestation levels from 5 to 35%. Both PCR assays confirmed all field seed samples to be infested with *A. burnsii*, amplifying PCR products of the expected size. The pathogen DNA quantities and Cq values in these seed samples ranged from 0.42 ng to 4.4 pg and 22.83 to 29.14, respectively.

Discussion

The current routine detection and identification of *A. burnsii* depends on visual examination of morphologic characters on infected seeds or culture media, which is time consuming and laborious. It also requires skill and technical expertise

in taxonomy. Hence, more rapid and sensitive methods than traditional culture methods need to control the disease by preventing the use of contaminated seed. This study aims to develop a sensitive and specific PCR based method for detection of *A. burnsii* in cumin seeds.

Molecular techniques have been widely used to investigate genetic relationships among *Alternaria* and related fungus species. Phylogenetic analysis of DNA or protein sequences has become a powerful tool for studying molecular taxonomy of *Alternaria* species (Pryor and Bigelow 2003; Hong et al. 2005; Udayashankar et al. 2012). In the present study, we targeted on *Alt a1* gene to study genetic relationships between *A. burnsii* and other *Alternaria* species-groups and to develop species-specific primers for the pathogen. The function of *Alt a1* gene is not completely known; however, the gene homologs are generally thought to be associated with plant pathogenicity (Cramer and Lawrence 2003). *Alt a1* gene of some *Alternaria* species was known to show greater sequence diversity than ITS, mt SSU and *gpd* gene (Cramer and Lawrence 2003; Pryor and Bigelow 2003). Our results indicated that *Alt a1* gene was quite useful to separate *A. burnsii* and the other species from each other. All pathogen isolates clustered into the same phylogenetic groups with high bootstrap values. Similarly, Lourenço et al. (2009) reported that *Alt a1* gene was

useful to reveal phylogenetic differences among *Alternaria solani* populations associated with potato and tomato plants. Hartevelde et al. (2013) grouped *Alternaria* species-groups associated with leaf blotch and fruit spot of apples into four phylogenetic clades based on sequence data of the endoPG and Alt a1 genes. Pavón et al. (2010) described PCR assays targeting Alt a1 gene for detection and identification of *Alternaria* species-groups in raw and processed foodstuffs.

Polymerase chain reaction is a rapid and sensitive method that commonly used for the detection and identification of plant pathogenic fungi. Species-specific PCR primers have been developed and used for PCR detection of *Alternaria* species in different hosts such as carrot, apple, sunflower, lilac and crucifers (Johnson et al. 2000; Konstantinova et al. 2002; Guillemette et al. 2004; Mmbaga et al. 2011; Udayashankar et al. 2012). In this study, two primer sets, Ab35/326 and AB177/403, were designed on the basis of alignment of Alt a1 gene sequences. Ab35/326 primers designed for conventional PCR allowed the amplification of 1 pg of pure genomic DNA, but did not amplify DNA extracts from healthy plant tissue and the other fungi species analyzed. The assay was able to differentiate from *A. burnsii* to *A. alternata* associated with cumin blight. The sensitivity of Ab35/326 primers was assessed by analysis of DNA samples prepared by mixing the powder of *A. burnsii*-infested seed and noninfested seed samples. PCR assay showed a detection limit of 0.2% infested samples. Also, the primer pairs confirmed the presence of *A. burnsii* in all field seed samples tested. This method allowed the detection of pathogen infection in cumin seeds as more rapid and sensitive compared with the cultural methods which required incubation for 10–15 days to induce the pathogen sporulation. Similar results were observed by Pryor and Gilbertson (2001), who developed a conventional PCR assay to allow the detection of the pathogen even in carrot seed samples with 0.1% infestation rate prepared by mixing known amounts of *A. radicina*-infested seed with noninfested seed. The PCR assay developed for the detection of *A. helianthi* in infected sunflower seed confirmed the presence of the pathogen in seed powder samples of 2% infestation level, but showed variable results with 1% infested samples (Udayashankar et al. 2012).

Real-time PCR has been known as a useful and sensitive tool for disease management, pathogen detection, evaluation of host–pathogen interactions, and phytosanitary screens. Compared to conventional PCR, real-time PCR is faster, more sensitive, less labor-intensive and does not require post handling of PCR product (Schena et al. 2004; Okubara et al. 2005). In this study, we designed a real-time PCR assay for detection and identification of *A. burnsii* infection in cumin seeds based on SYBR Green I technology, which is a simpler and lower-cost method compared to other sequence specific (Molecular beacons, TaqMan and

Scorpion probes) methods (Schena et al. 2004). The real-time PCR assay was highly specific, detecting 0.1 pg of pathogen DNA. Also, the assay appeared to be more sensitive than the conventional PCR assay and allowed the detection of the pathogen DNA in seed samples with 0.1% infestation. The standard curves ($r^2=0.99$) confirmed the linearity of the quantification process between exponential increases in fungal DNA quantities and cycle threshold values. The results indicated that real-time PCR is an ideal tool for confirming the pathogen infection in field seed samples. Similarly, Guillemette et al. (2004) described conventional and real-time PCR assays on the basis of the nucleotide sequence of ATP-binding cassette (ABC) transporter gene and the nonribosomal peptide synthase (NRPS) gene for detecting *Alternaria brassicae* in cruciferous seed. Real-time PCR method developed was more sensitive than the conventional PCR, and confirmed the presence of the pathogen DNA in seed samples with 2% *A. brassicae* infection. Real-time PCR assay described by Leiminger et al. (2015) allowed to differentiate *A. solani* and *A. alternata* and quantify *Alternaria* colonization in infected potato leaves during early disease development. Kordalewska et al. (2015) developed PCR and real-time PCR assays that allowed species-specific detection of *A. alternata* on the basis of alignment of β -tubulin gene sequences. Similarly, several authors have been described real-time PCR assays for detection of *Alternaria* species (Kumar et al. 2013; Singh et al. 2014).

In conclusion, we described conventional and real-time PCR assays on the basis of sequence alignment of Alt a1 gen for the detection and identification of *A. burnsii* that is an important fungal pathogen restricting cumin production of Turkey. The PCR assays developed in this study will be a useful tool for the detection of *A. burnsii* infection in cumin seed and will contribute to the development of effective disease control methods to this pathogen.

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Conflict of interest G. Özer and H. Bayraktar declare that they have no competing interests.

References

- Cramer RA, Lawrence CB (2003) Cloning of a gene encoding an Alt a 1 isoallergen differentially expressed by the necrotrophic fungus *Alternaria brassicicola* during Arabidopsis infection. *Appl Environ Microbiol* 69(4):2361–2364
- Divakara Sastry E, Anandaraj M (2013) Cumin, fennel and fenugreek. Soils, plant growth and crop production. *Encyclopedia of Life Support Systems (EOLSS)*, p 1–10
- Guillemette T, Iacomi-Vasilescu B, Simoneau P (2004) Conventional and real-time PCR-based assay for detecting pathogenic *Alternaria brassicae* in cruciferous seed. *Plant Dis* 88(5):490–496

- Harteveld DO, Akinsanmi OA, Drenth A (2013) Multiple *Alternaria* species groups are associated with leaf blotch and fruit spot diseases of apple in Australia. *Plant Pathol* 62(2):289–297
- Holiday P (1980) *Fungus diseases of tropical crops*. Cambridge University Press, Cambridge
- Hong SG, Cramer RA, Lawrence CB, Pryor BM (2005) Alt a 1 allergen homologs from *Alternaria* and related taxa: analysis of phylogenetic content and secondary structure. *Fungal Genet Biol* 42(2):119–129
- Johnson RD, Johnson L, Kohmoto K, Otani H, Lane CR, Kodama M (2000) A polymerase chain reaction-based method to specifically detect *Alternaria alternata* apple pathotype (A. mali), the causal agent of *Alternaria* blotch of apple. *Phytopathology* 90(9):973–976
- Konstantinova P, Bonants PJ, Van Gent-Pelzer MP, Van Der Zouwen P (2002) Development of specific primers for detection and identification of *Alternaria* spp. in carrot material by PCR and comparison with blotter and plating assays. *Mycol Res* 106(01):23–33
- Kordalewska M, Brillowska-Dąbrowska A, Jagielski T, Dworecka-Kaszak B (2015) PCR and real-time PCR assays to detect fungi of *Alternaria alternata* species. *Acta Biochim Pol* 62(4):707–712
- Kumar S, Singh R, Kashyap PL, Srivastava AK (2013) Rapid detection and quantification of *Alternaria solani* in tomato. *Sci Hortic* 151:184–189
- Lawrence DP, Gannibal PB, Peever TL, Pryor BM (2013) The sections of *Alternaria*: formalizing species-group concepts. *Mycologia* 105(3):530–546
- Leiminger J, Bäßler E, Knappe C, Bahnweg G, Hausladen H (2015) Quantification of disease progression of *Alternaria* spp. on potato using real-time PCR. *Eur J Plant Pathol* 141(2):295–309
- Lourenço V Jr, Moya A, González-Candelas F, Carbone I, Maffia LA, Mizubuti ES (2009) Molecular diversity and evolutionary processes of *Alternaria solani* in Brazil inferred using genealogical and coalescent approaches. *Phytopathology* 99(6):765–774
- Mmbaga MT, Shi A, Kim MS (2011) Identification of *Alternaria alternata* as a causal agent for leaf blight in *Syringa* species. *Plant Pathol J* 27(2):120–127
- Okubara PA, Schroeder KL, Paulitz TC (2005) Real-time polymerase chain reaction: applications to studies on soilborne pathogens. *Can J Plant Pathol* 27(3):300–313
- Özer G, Bayraktar H (2015) Determination of fungal pathogens associated with *Cuminum cyminum* in Turkey. *Plant Prot Sci* 51:74–79
- Pavón MÁ, González I, Pegels N, Martín R, García T (2010) PCR detection and identification of *Alternaria* species-groups in processed foods based on the genetic marker Alt a 1. *Food Control* 21(12):1745–1756
- Pryor BM, Bigelow DM (2003) Molecular characterization of *Embelisia* and *Nimbya* species and their relationship to *Alternaria*, *Ulocladium* and *Stemphylium*. *Mycologia* 95(6):1141–1154
- Pryor BM, Gilbertson RL (2001) A PCR-based assay for detection of *Alternaria radicina* on carrot seed. *Plant Dis* 85(1):18–23
- Sambrook J, Fritsch EF, Maniatis T (1989) *Molecular cloning: a laboratory manual*, 2nd edn. Cold Spring Harbor Laboratory Press, New York
- Schena L, Nigro F, Ippolito A, Gallitelli D (2004) Real-time quantitative PCR: a new technology to detect and study phytopathogenic and antagonistic fungi. *Eur J Plant Pathol* 110(9):893–908
- Simmons EG (2007) *Alternaria: an identification manual*. CBS Fungal Diversity Centre, Utrecht
- Singh R, Kumar S, Kashyap PL, Srivastava AK, Mishra S, Sharma AK (2014) Identification and characterization of microsatellite from *Alternaria brassicicola* to assess cross-species transferability and utility as a diagnostic marker. *Mol Biotechnol* 56(11):1049–1059
- Tamura K, Stecher G, Peterson D, Filipski A, Kumar S (2013) MEGA6: molecular evolutionary genetics analysis version 6.0. *Mol Biol Evol* 30(12):2725–2729
- Udayashankar AC, Nayaka SC, Archana B, Anjana G, Niranjana SR, Mortensen CN, Lund OS, Prakash HS (2012) Specific PCR-based detection of *Alternaria helianthi*: the cause of blight and leaf spot in sunflower. *Arch Microbiol* 194(11):923–932

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